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Jessica N. Reiners

*South Dakota State University*

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LYSINE BIOAVAILABILITY OF TWO LIPID COATED LYSINE PRODUCTS  
AFTER EXPOSURE TO SILAGES WITH DIFFERENT ACIDITY

BY

JESSICA N. REINERS

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Animal Science

South Dakota State University

2017

LYSINE BIOAVAILABILITY OF TWO LIPID COATED LYSINE PRODUCTS  
AFTER EXPOSURE TO SILAGES WITH DIFFERENT ACIDITY

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Animal Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

~~Jeff Held, Ph.D.~~  
Derek Brake, Ph.D.  
Thesis Advisor

Date

~~Jeff Held, Ph.D.~~  
Jeff Held, Ph.D.  
Thesis Advisor

Date

~~Joseph Cassady, Ph.D.~~  
Joseph Cassady, Ph.D.  
Head, Department of Animal Science

Date

~~Dean~~  
Dean, Graduate School

Date

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## ABBREVIATIONS

AA	Amino acid
ADF	Acid detergent fiber
Ala	Alanine
Asn	Asparagine
Asp	Aspartate
Arg	Arginine
BW	Body weight
CP	Crude protein
Cys	Cysteine
d	Day
DM	Dry matter
DMB	Dry matter basis
DMI	Dry matter intake
EE	Ether extract
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
h	Hour
His	Histidine
HPLC	High performance liquid chromatography
Ile	Isoleucine
Leu	Leucine

Lys	Lysine
Lys-HCl	Lysine hydrochloride
ME	Metabolizable energy
ME <sub>m</sub>	Metabolizable energy for maintenance
Met	Methionine
mM	Millimolar
MP	Metabolizable protein
N	Nitrogen
NDF	Neutral detergent fiber
OM	Organic matter
Phe	Phenylalanine
Pro	Proline
RDP	Rumen degradable protein
RP	Ruminally protected
RPAA	Ruminally protected amino acids
RPLys	Ruminally protected lysine
RUP	Rumen undegradable protein
SEM	Scanning electron microscope
Ser	Serine
Thr	Threonine
TMR	Total mixed ration
Trp	Tryptophan
Tyr	Tyrosine



Val

Valine

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## ABSTRACT

LYSINE BIOAVAILABILITY OF TWO LIPID COATED LYSINE PRODUCTS  
AFTER EXPOSURE TO SILAGES WITH DIFFERENT ACIDITY

JESSICA N. REINERS

2017

Three experiments were conducted to determine bioavailability of 2 lipid coated Lys products (EB and EC). In an initial experiment, Lys disassociation from 2 lipid coated Lys products was best described by a first-order kinetic model after incubation in alfalfa- or corn-silage at 2 different amounts of acidity. Greater amounts of Lys immediately disassociated from EC (15.7%) than EB (4.9%;  $P < 0.05$ ). Lysine that initially disassociated in corn silage at low pH (0.5%) was less than corn silage at a more neutral pH (18.7%), whereas Lys that initially disassociated in alfalfa silage was not affected ( $P = 0.26$ ) by pH. Lysine that slowly disassociated from lipid coated Lys differed by product ( $P = 0.01$ ), and acidity ( $P < 0.01$ ) affected amounts of Lys that slowly disassociated differently within alfalfa- or corn-silage ( $pH \times \text{silage} = 0.02$ ). Greater amounts of Lys disassociated from acidic corn silage (79%) than neutral corn silage (32%;  $P = 0.01$ ). However, amounts of Lys that slowly disassociated from alfalfa silage was not impacted by acidity ( $P = 0.40$ ). Additionally, amounts of Lys that did not disassociate ( $P < 0.04$ ) within 24 h were greater ( $P < 0.04$ ) when lipid coated Lys was mixed with neutral silages (44%) compared to acidic silages (21%). However, rate of Lys disassociation from lipid coated Lys products was not affected by acidity or exposure to alfalfa- or corn-silage ( $P = 0.21$ ).

In a second experiment, we measured in vitro ammonia release from EB and EC after mixing with alfalfa- or corn-silage at different amounts of acidity with or without monensin. Ammonia release from Lys increased (*Quadratic* < 0.01) with greater amounts of time; however, differences between in vitro cultures with added Lys and negative control were not detected until 36 h of incubation. By 36 h, all cultures containing added Lys were greater than the negative control. After 42 h of incubation, ammonia release from Lys was greatest from EC and when lipid and Lys-HCl in amounts identical to EB were provided to cultures; EB, Lys-HCl, and lipid and Lys in amounts identical to EC were intermediate. After 48 h of incubation, ammonia release from Lys in EB was greatest and Lys-HCl was least, but EC and amounts of lipid and Lys-HCl identical to either EB or EC were intermediate. Ammonia release from Lys were less from cultures containing monensin after 48 h of incubation.

In experiment 3, 9 white-faced ewes ( $70.1 \pm 5.2$  kg BW;  $5.3 \pm 0.6$  yr) were used to measure Lys bioavailability by a slope-ratio analysis from 2 lipid coated Lys products and Lys-HCl after mixing in corn silage. Plasma Lys concentrations increased (*Linear* < 0.01) in response to abomasal infusion of Lys, and bioavailability of EB, EC, and Lys-HCl were calculated to be 10.74%, 18.82%, and 11.87%, respectively. However, increases in plasma Lys from EB (*Linear* = 0.41) and Lys-HCl (*Linear* = 0.36) were not different from plasma Lys supported by diet alone, but the rate of increase in plasma Lys in response to EC tended (*Linear* = 0.15) to be greater than plasma Lys levels from diet. Evidently, manufacturing method among lipid coated Lys products as well as physical and chemical characteristics of diet can impact availability of Lys from lipid coated Lys products.

Key words: acidity, cattle, lysine, silage

# **CHAPTER I**

## **Literature Review**

## EFFICIENCY OF PROTEIN AND AMINO ACID UTILIZATION IN RUMINANTS

### *Nitrogen efficiency and use in ruminants*

Ruminants are less efficient at utilizing dietary nitrogen (N) than non-ruminants (Kohn et al., 2005), and N efficiency can range from 10-40% (Calsamiglia et al., 2010). Thus, it is not surprising that a large amount of effort has concentrated on an improved understanding of N efficiency in ruminants (Kohn et al., 2005; Huhtanen and Hristov, 2009; Calsamiglia et al., 2010); however, there has been little apparent increase in efficiency of N utilization by ruminants (Calsamiglia et al., 2010). A lack of improvement in efficiency of N use by ruminants has led at least one author (Tamminga, 1992) to speculate that the greatest contribution to inefficient use of N is ruminal metabolism.

Law et al. (2009) evaluated N efficiency at 3 different dietary CP levels (114, 144, and 173 g/kg DM) in lactating cows. During early lactation, N efficiency increased (i.e. 35 to 42%) as dietary CP levels decreased. When CP levels were changed for late lactation, N efficiency decreased (i.e. 37 to 30%) when CP increased (i.e. from 144 to 173 g/kg DM), and increased (i.e. 30 to 36%) when CP decreased (i.e. from 173 to 144 g/kg DM). Additionally, Huhtanen and Hristov (2009) reported a 10% decrease in milk yield among lactating cows fed a 144 g CP/kg DM compared to lactating cows fed 173 g CP/kg DM. Further, reductions in diet CP from 173 g CP/kg DM to 144 g CP/kg DM decreased milk yield (7%) over the entire lactation (Huhtanen and Hristov, 2009).



### ***Protein and amino acid requirements of lactating dairy cows***

Current predictions of protein requirements among lactating cows are based on milk protein production (NRC, 2001); however, predictions of protein requirements among cows are often imprecise because measures of protein do not account for AA requirements. Indeed, protein and AA utilization can be improved when cows are fed diets with an AA profile that more nearly matches AA requirements for maintenance, growth, or lactation (Fraser et al., 1991). Unfortunately, specific AA requirements of lactating cows are not known (NRC, 2001). Therefore, the goal of protein and AA nutrition is to maximize the efficient utilization of dietary N to provide optimal milk production per unit of consumed N (Schwab, 1995). Even though a precise understanding of all AA requirements among lactating cows has yet to be elucidated, Lys has been established as one of the most limiting AA in corn-based diets (Schwab, 1995; NRC, 2001; Vyas and Erdman, 2009), and Lys requirements of lactating cows have been estimated to be 7.08% of metabolizable protein (MP; NRC, 2001).

### ***Crude protein and metabolizable protein***

Dietary CP can optimize milk production at amounts near 23% but predictions of milk production based on CP are variable (NRC, 2001). Increasing dietary CP in diets fed to lactating cows often decreases N efficiency (Huhtanen and Hristov, 2009) and increases cost of production (Lee et al., 2012). Further, greater dietary CP levels may reduce reproductive efficiency in lactating cows (NRC, 2001).

Protein requirements of ruminants can be estimated by several different systems. The CP system assumes that all protein contains 16% N, and CP requirements are

estimated in amounts needed to optimize ruminal fermentation. Thus, the CP system divides measures of CP into amounts of CP that are ruminally degradable (RDP) and amounts of CP that are ruminally undegradable (RUP). Ruminally degradable protein represents N sources (i.e., ammonia, AA, protein) available to ruminal microbes for maintenance and growth. Subsequently, ruminal microbes pass to the small intestine and supply a large amount of absorbable AA to the animal as MCP (NRC, 2001). Generally, MCP is sufficient to meet the AA needs for lactating cows (Virtanen, 1966). Supplementation of RUP may decrease the flow of MCP into the duodenum and modify amounts of AA that flow to the small intestine (Santos et al., 1998).

The MP system is a measure of protein available to ruminants for the purposes of maintenance, gain, and lactation. Metabolizable protein is comprised of MCP and AA available from RUP but is intestinally absorbed. Microbial protein is a high-quality protein source due to its high digestibility and the AA composition of MCP (Storm and Ørskov, 1983) is like the AA profile of lean body tissue (Ainslie et al., 1993) and milk (Jacobson et al., 1970; McCance and Widdowson, 1978; Waghorn and Baldwin, 1984).

Like the CP system, the MP system is also inadequate at accurately predicting protein requirements of cattle because the MP system does not consider specific AA requirements. Optimizing efficiency of protein utilization (i.e., N efficiency) and reducing N excretions will be difficult with any protein system that does not accurately account for specific AA requirements of various physiological functions because it is unlikely that AA profiles will ideally match requirements.

### ***Limiting amino acids***

Animals require AA to meet requirements for protein synthesis to support lactation, growth, or maintenance purposes. Indeed, MP is most efficiently used for protein synthesis when the AA profile of MP matches the AA requirements (NRC, 2001).

Classically, AA have been categorized as essential and non-essential AA. Historically, AA synthesized in adequate amounts to support protein synthesis for maintenance and growth in vivo have been termed as non-essential AA (Wu et al., 2013). Typically, it is not necessary to supplement non-essential AA to diets. Conversely, essential AA have been described as AA not synthesized in sufficient quantities to meet requirements for maintenance and growth (Wu et al., 2013). Therefore, it is often necessary to supply essential AA to animals via the diet.

Amino acids that limit protein synthesis in the animal can be described as limiting AA. First limiting AA are those that are found in the smallest quantities relative to the requirements for maintenance or productive purposes, and will limit production responses until the requirement is met. Milk protein synthesis is often most limiting by Met and Lys (Rulquin and Verite, 1993; Schwab et al. 1993; NRC, 2001) among cows fed corn-based diets. Lysine often first-limits milk protein synthesis because Lys is found in lesser amounts in most conventional feed proteins (Schwab, 1995). Storm and Ørskov (1984) reported that efficiency of N use among lambs infused with ruminal microorganisms and no added Lys was less compared to lambs provided ruminal microorganisms and additional Lys. Further, N efficiency was less when lambs were limited by Lys in comparison to other AA, suggesting that Lys first-limits N retention in sheep (Storm and Ørskov, 1984). Ostensibly, changes in diet have little effect on the AA

composition of MCP (Satter and Roffler, 1975; Storm et al., 1983), and Russell (2005) reported that added metabolizable Lys in addition to MCP will increase milk production.

Cattle fed corn- and corn-silage based diets are first-limited by metabolizable Lys (King, 1991; Polan, 1991; Schwab, 1992), and most feed ingredients have less amounts of Lys than amounts of Lys typically provided by MCP (NRC, 2001). Several authors (Chung et al., 2006; Wu, 2009) have reported that crystalline Lys may be poorly degraded in the rumen and may pass into the small intestine to be absorbed. However, others (Chalupa, 1976; Onodera, 1993; Russell, 2006) have reported that crystalline Lys added to diet does not increase metabolizable Lys (Wu and Papas, 1997). Therefore, Lys added to the diet should be resistant to ruminal degradation and still available for absorption in the small intestine to increase metabolizable Lys flow.

Increasing metabolizable Lys to lactating cows fed corn-based diets augments milk protein content and yield; however, these results are not consistent (NRC, 2001). The lack of a consistent response may be due to another AA limiting performance. Rulquin (1993), Schwab (1996) and Sloan et al. (1998) reported that increases in milk protein production are more predictable when Lys supply in MP is increased and other AA in MP meet or are near estimated requirements for cattle. Improving the metabolizable AA profile to lactating cows would increase N efficiency and may allow for diets lower in CP to be fed.

### ***Ruminal protection technology and ruminally protected amino acids***

Noel (2000) described ruminally protected feeds as, “nutrient(s) fed in such a form that provides an increase in the flow of that nutrient(s), unchanged, to the

abomasum, yet is available to the animal in the intestine.” Proteins or AA that are protected from ruminal degradation and proteins with high ruminal escape characteristics can supply additional AA that most limit production.

Several authors (Kirby et al., 1983; Veira et al., 1991; Schwab, 1995) have posited that lactating cows are reliant on dietary proteins with a greater content of digestible RUP when most or all the forage in the diet is supplied by grasses and legumes. Approximately 80% of N in alfalfa is true protein, but when alfalfa is ensiled the true protein content decreases to 50% (Satter and Roffler, 1975). Often, the basal diet fed to lactating cows contains adequate RDP for MCP synthesis, but not enough RUP to match limiting AA requirements (NRC, 2001).

### ***Protein protection methods and efficacy***

Addition of ruminally protected proteins to diets has been evaluated for use when MCP is not adequate to meet AA needs. A myriad of techniques designed to protect proteins from ruminal degradation have been evaluated (Broderick et al., 1991). These methods must not only protect proteins from degradation in the rumen, but cannot cause significant, deleterious changes to intestinal digestibility and availability of amino acids. Moreover, protection methods must be safe for consumption by animals and handling by humans, and cost-effective.

Heat treatment is among the oldest methods used to protect protein from ruminal degradation (Broderick et al., 1991; Schwab et al., 1995) and heat treatment remains the most common method to limit ruminal degradation of protein in North America (NRC, 2001). Heating protein to reduce ruminal degradation works primarily by synthesis of

Maillard products. Maillard products are the result of reaction of free sugars (i.e., glucose or fructose) and lysine. When adequate heat is applied, free sugars and Lys react to form a Schiff's base, ultimately resulting in an Amadori compound. A Schiff base is a reversible reaction and these compounds are nutritionally available to animals; however, conversion of a Schiff base to an Amadori compound is an irreversible reaction, and protein within an Amadori compound is not digested by mammalian enzymes (Broderick et al., 1991).

Heat treatment of proteins can have deleterious effects on protein quality, because heating proteins reduces not only ruminal degradation of protein, but also intestinal digestibility. Under-heating proteins may cause damage to some AA and may not effectively protect the protein being treated (NRC, 2001; Schwab, 1995), allowing for more ruminal degradation than adequately heated protein. Overheating will also decrease the AA contained within the feed, most of all Lys, which is the AA most susceptible to heat treatment (Broderick et al., 1991). Further, overheating will negatively impact the digestibility of these feeds when they reach the small intestine.

Another method often used to protect feed proteins from ruminal degradation is chemical treatment. Chemical protection can decrease ruminal protein degradation with little impact on small intestinal absorption (Schwab, 1995). Indeed, several methods of chemical protection have been reported in the literature (e.g., aldehydes, acids, alkalis, ethanol).

Aldehydes can form chemical cross-linkages (i.e., Schiff's base) with Lys that prevent microbial proteolysis of protein. Initially, interest in chemical treatment of protein to reduce ruminal degradation involved treatment of high-quality proteins (e.g.,

casein, oilseed meals) with formaldehyde, but formaldehyde also offers opportunity to preserve feed proteins during ensiling (Tamminga, 1979; Schwab, 1995). Typically, forage proteins are hydrolyzed during fermentation and the subsequent ensiled feed often has less protein compared to the pre-ensiled forage (Schwab, 1995). Formaldehyde can augment protein content of ensiled feed and prevent microbial proteolysis during ensiling and subsequently ruminal fermentation (Schwab, 1995). Overall, application of formaldehyde to protect proteins limits ruminal degradation and increases N retention and protein digestion in the small intestine (Ferguson, 1975). However, optimal rates of formaldehyde application are not well understood (Schwab, 1995) and may therefore vary between feed ingredients. Formaldehyde application rates are further obfuscated by innate characteristics of feed ingredients (i.e., RDP, moisture, carbohydrate content and particle size; Kaufmann and Lüpping, 1982). Formaldehyde treatment of feed can also limit MP. Kaufmann and Lüpping (1982) reported that formaldehyde application to soybean meal at a rate of 2 g/kg of DM decreased N solubility in a phosphate buffer in a curvilinear response, but application of formaldehyde from 1.5 to 5.0 g/kg DM decreased intestinal digestibility of protein in sheep from 85 to 30%. Currently, use of formaldehyde in feed manufacture is limited by the United States Environmental Protection Agency because of human health concerns (Broderick et al., 1991; Schwab, 1995). Additionally, protection of proteins with formaldehyde is not viable due to inadequate knowledge of required application rates for various feed ingredients.

Other chemical processing techniques that have been investigated as methods to mitigate ruminal degradation of AA include acid or alkali treatment of feeds. Acids and alkalis can denature protein and subsequently limit protein hydrolysis by ruminal

microflora (Schwab, 1995). During ensiling, acids will cause a rapid drop in pH and limit proteolysis. However, acids do not protect silage proteins in the rumen. Therefore, acid treatment is often coupled with aldehyde treatment to ensure ruminal protection (Broderick, 1991; Schwab, 1995). Treatment of feed protein with acids or alkalis is beneficial in that it does not have adverse effects on the availability or absorbability of AA when they reach the intestine. Mir et al. (1984) reported an increase in N retention in calves and lactating cows fed soybean meal protected with sodium hydroxide. Additionally, lactating cows fed sodium hydroxide treated soybean meal produced more milk than control cows with no concomitant increase in dry matter intake (Mir et al., 1984). Waltz and Loerch (1986) evaluated acid or alkali treated soybean meal in situ and evaluated N remaining after 4, 8, 12 or 24 h. Soybean meal that was treated with hydrochloric acid or sodium hydroxide and air-dried retained greater amounts ( $P < 0.05$ ) of N when compared to the control soybean meal and exhibited a much lesser ( $P < 0.05$ ) rate of N loss (Waltz and Loerch, 1986). Soybean meal dried at 100 °C also had greater ( $P < 0.05$ ) N retention up to 24 h when compared to the untreated, undried controls (Waltz and Loerch, 1986). In addition to in situ evaluation, Waltz and Loerch (1986) also tested acid and alkali protection in a digestibility trial using lambs. Lambs receiving soybean meal that was protected with a weak acid (i.e., 5% acetic or propionic acid) did not show differences from control fed lambs in N digested, retained, or excreted via urine (Waltz and Loerch, 1986).

Tannins are a secondary plant defensive compound innate to a myriad of forages. Tannins form hydrogen bonds with protein, and several investigators (Leroy et al., 1965; Driedger and Hatfield, 1972) have evaluated the ability of exogenous tannins to mitigate



ruminal degradation of AA. Tannins are broadly classified as hydrolysable tannins and condensed tannins (Broderick et al., 1991). Generally, tannins are thought to associate with feed protein in the rumen but to disassociate in the acidic conditions of the abomasum. Usually, tannin disassociation is best with condensed tannins because condensed tannins are more stable, less toxic, and has a more favorable relationship between pH of digesta and tannin protein binding ability compared to hydrolysable tannins (Broderick, et al., 1991). After tannins disassociate from protein in the abomasum, proteins contribute to the metabolizable AA pool. Additionally, tannin protein bonds are hydrophobic. Therefore, tannins offer a method of feed processing able to mitigate ruminal degradation of feed AA without formation of Maillard products or aldehydes. However, tannins can also bind non-feed protein in ruminal digesta. Moreover, inclusion of tannins at greater than 4% diet DM reduce feed intake and limit ruminal fermentation by apparently limiting amounts of ruminally available N to microbes (Barry and Duncan, 1984). More data are needed to identify condensed tannins best able to mitigate ruminal degradation of AA without concomitant deleterious effects on ruminal fermentation (Waghorn and Shelton, 1992) before tannins can be precisely used in feed processing to improve metabolizable AA flow from feed in ruminant diets.

Physical treatments of feed can also be used to prevent ruminal degradation of AA. Unlike chemical and heat treatment, physical treatment of protein does not interact with specific AA. Thus, successful physical protection of AA creates opportunity to provide metabolizable AA identical to AA added to the diet. Methods to physically protect feeds include lipid coatings and use of pH sensitive polymeric coating. Lipid coating offers opportunity to prevent ruminal degradation of AA by inhibiting ruminal

microbial fermentation (Schingoethe, 1988). Lynch et al. (1987) reported that using cottonseed oil to protect soybean meal decreased protein degradation in situ; a similar result was found when proteins were encapsulated with calcium soaps (Sklan, 1989).

### ***Current ruminal protection technologies***

Technologies that allow ruminal protection of individual AA include low solubility analogues, lipid coating, and pH sensitive polymeric coating. The protection of single AA instead of proteins can allow more targeted supplementation of AA (Met, Lys) to meet needs for metabolizable AA. Meeting individual AA requirements can decrease excess N intake and potentially increase N efficiency. Currently, a myriad of ruminally protected Lys products are commercially available in the United States (Table 1.1).

Hydroxymethyl Lys (HML) is a Lys analog with limited ruminal solubility. Hydroxymethyl Lys is created by mixing formaldehyde with Lys-HCl in an aqueous solution of calcium hydroxide, and HML is believed to be protected from ruminal degradation but available in the small intestine after being acidified in the abomasum (Bertram et al., 1978). However, Kenna and Schwab (1981) reported no increase in milk production among cows supplemented HML. These authors (Kenna and Schwab, 1981) posited that diet acidity may have impacted Lys availability. Further, Wu and Papas (1997) found that small intestinal absorption of Lys analogs was limited and that increased levels of dietary fat from HML can decrease ruminal function by inhibiting ruminal microbial fermentation (Schingoethe, 1988).

Wu and Sandhu (1986) evaluated encapsulating Lys with saturated fatty acid. These authors (Wu and Sandhu, 1986) mixed Lys with water and binding agents, then

extruded or rolled the mixture to create pellets that were then dried. Subsequently, pellets were coated with protective coating material (Wu and Sandhu, 1986). Lipids used in lipid coating are often inexpensive food-grade fats, such as palmitate, and rely on the resistance of lipids to ruminal degradation as well as their ability to be digested in the small intestine, making the Lys core available (Wu and Papas, 1997). Encapsulation in lipid has varied success and is known to deliver lower amounts of Lys than expected. Wu and Papas (1997) found that the Lys availability in the small intestine is inversely related to degree of ruminal protection. Several authors (Bateman et al., 1999; Arriola Apelo et al., 2014; Lee et al., 2015) have reported little to no performance responses to lactating cows supplemented with lipid coated ruminally protected Lys (RPLys). Bateman et al. (1999) examined the effect of RPLys supplemented to lactating cows fed soybean meal or urea as the primary N source. Milk fat, protein, and yield did not differ between any dietary treatments (Bateman et al., 1999). A study by Arriola Apelo et al. (2014) reported that lactating cows supplemented with RPLys did not increase milk protein or yield, N efficiency, or plasma Lys concentration over control cows. Lee et al. (2015) reported that milk yield, protein and fat, and plasma Lys concentrations were not different among lactating cows supplemented RPLys when compared to control cows. A lack of response in these studies (Bateman et al., 1999; Arriola Apelo et al., 2014; Lee et al., 2015) may be due to an inverse relationship between protection and metabolizable Lys. Ji et al. (2016) reported that Lys disassociated from lipid coated Lys after mixing in diets. Other authors have theorized that diet acidity may impact Lys availability as well (Wu and Papas, 1997; Ji et al., 2016).

Another method currently used to protect individual AA is a pH-sensitive polymeric coating. This coating is sensitive to the pH of the abomasum and small intestine, but resistant to degradation in the less acidic rumen. Ruminally protected AA products are by design sensitive to degradation in low pH environments. Thus, acidity of the silage may prematurely degrade the polymeric coating and expose the coated AA to the ruminal environment.

### ***Resistance of ruminally protected Lys to Lys loss***

Few reports are available on effect of diet characteristics on efficacy of RPLys. Ji et al. (2016) evaluated impacts of mixing on Lys that remained associated from 6 commercially available RPLys. Ji et al. (2016) reported that when RPLys was mixed in TMR,  $16.4 \pm 4.7\%$  Lys immediately disassociated from all products and that after 24 h of incubation in feed nearly 60% of Lys disassociated from all RPLys. Results from Ji et al. (2016) suggest that forces of mixing RPLys in the diet will reduce available Lys from RPLys.

Several authors (Wu and Papas, 1997; Ji et al., 2016) suggested that diet acidity may impact RPLys efficacy, but few studies have evaluated effects of diet acidity on RPLys (Polan et al., 1991). Wu and Papas (1997) hypothesized that the acidic characteristics of corn-silage may perforate the surface of RPLys after mixing. Further, these authors (Wu and Papas, 1997) speculated that the Lys core could then be subject to solubilization by water molecules that breach the coating.

### ***Acid-catalyzed hydrolysis***

Acid can catalyze the hydrolysis of triglycerides to free fatty acids and glycerol (Bender et al., 1961; Carey, 2003) and this hydrolysis is promoted in environments with large concentrations of water (Le Chatelier, 1884; Bender et al., 1961, Carey, 2003). It is possible that diets containing acidic ingredients or ingredients with large amounts of water (e.g., ensiled feed) may have an impact on lipid coated RPLys. It is possible that the lipid coating of RPLys may be hydrolyzed when exposed to acidic diets with appreciable amounts of moisture. Further, it is reasonable that Lys contained in lipid coated RPLys will then be subject to solubilization.

### *In vitro analysis*

Lysine can be deaminated in the rumen by microbes for maintenance and growth (Russell, 2006). Thus, it is important that RPLys be tested in ruminal fluid to determine resilience of coating to the ruminal environment. Elwakeel et al. (2012) reported that HML produced less ammonia (mean = 2.2 mM) than Lys-HCl (mean = 54.5 mM) in vitro. Importantly, however, these authors (Elwakeel et al., 2012) reported that low Lys degradation among HML was not due to protection from hydrolysis by microbial enzymes, but rather due to an inhibitory effect of HML on overall microbial fermentation in vitro. Chung et al. (2006) reported that in vitro inoculations with Lys-HCl showed minimal ammonia production (mean = 6.7 mg/dL) when continuous culture fermenters received added Lys to diets in amounts of 0%, 1.03%, or 2.06% on a DMB. However, Chung's ammonia levels are not reported by time, it is therefore unclear if the ammonia values were averaged across time points or what time point was used.

### ***Monensin and protein sparing***

Monensin decreases fecal nitrogen and increases nitrogen digestibility in the lower digestive tract by 5%, possibly due to a decrease in ruminal degradation of protein and amino acid (i.e., protein sparing effect; Ruiz et al, 2001). Ruminal bacteria that are sensitive to monensin are gram positive and are known for rapid fermentation of Lys (Chen and Russell, 1989). In addition to these bacteria, monensin has been found to affect *Fusobacterum necrophorum* utilization of Lys. *Fusobacterum necrophorum* is resistant to monensin, but its uptake of Lys as a substrate is inhibited by monensin. Thus, it is plausible that inclusion of monensin in diets can reduce ruminal degradation of Lys (Russell, 2006). *Fusobacterum necrophorum* has been isolated from the ruminal fluid of cattle fed a dairy ration and was found to degrade supplied Lys (50mM) within 24 h in an in vitro system (Russell, 2006). Therefore, feeding both monensin and a RPLys product may decrease Lys degradation in the rumen and increase metabolizable Lys in the post-ruminal environment.

### ***Plasma responses to ruminally protected lysine***

In vitro and in vivo studies have been used to determine how well RPLys can withstand ruminal conditions. However, Rulquin and Kowalczyk (2003) suggested that using plasma AA concentration can be more accurate than in vitro estimates. Supplemented Lys that meets or exceeds the animals' need for Lys will typically cause an increase in plasma Lys when compared to non-supplemented animals. Oke et al. (1986) reported that RPLys increased plasma Lys content and N retention in sheep. Giallongo et al. (2016) reported that plasma Lys levels increased to levels equal to plasma Lys

concentration of cows fed a MP-adequate diet when cows fed a MP-deficient diet supplemented with 130 g/d of RPLys. Veira et al. (1991) reported that growing cattle supplemented with both RPLys and RPMet exhibited an increase in plasma Lys concentrations.

Conversely, Robinson et al. (2010) reported that lactating cows fed RPLys did not exhibit an increase in plasma Lys concentrations, suggesting that RPLys failed to meet Lys needs of cattle or its supplementation caused another AA to be most limiting. In another study, Robinson et al. (2011) reported that plasma Lys was not affected in early- or mid-lactation cows fed 94.4 g/d of RPLys. These authors (Robinson et al., 2011) concluded that a lack of response in plasma Lys was likely related to supplemental Lys failing to meet requirements for milk production. Similarly, Swanepoel et al. (2010) reported that neither plasma Lys, milk fat nor milk protein increased among lactating cows fed an RPLys. These 3 studies all used a lipid coated RPLys and mixed it with a TMR containing large quantities of silage. It is possible that the integrity of the RPLys may have been compromised and may explain the lack of response.

These mixed results suggest that effectiveness of RPLys products depends on numerous factors, such as method of manufacture, characteristics of the diet, and times of exposure to feed and ruminal microflora.

## **CONCLUSIONS**

Different methods have been attempted to meet AA requirements of cattle; however, a perfect method to ruminally protect AA remains elusive. Protection of proteins does not always provide adequate amounts of metabolizable Lys to the animal,

and oversupply of other AA can decrease N efficiency. Increases in specific metabolizable AA to cattle can increase the metabolizable AA profile to be nearer to an ideal protein and, increase N efficiency in cattle. However, response to ruminally protected AA products have been variable. Few data are available on effects of diet (e.g., mixing with feed, mastication) on efficacy of protected AA products.



**Table 1.1.** Commercially available ruminally protected lysine products

Name	Company of Production	Amount of Lys, %	Protection Method
AjiPro-L	Ajinomoto	40.0	Lipid
AminoShure-L	Balchem	45.0	Lipid
Bovi-Lysine	QualiTech	47.0	Lipid
LysiPEARL	Kemin Industries	47.5	Lipid
Megamine-L	Arm & Hammer	20.0	Lipid
MetaboLys	H. J. Baker & Bro.	32.0	Lipid
USA Lysine	Kemin Industries	65.0	Lipid

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**CHAPTER II**  
**LYSINE BIOAVAILABILITY AMONG TWO LIPID COATED LYSINE**  
**PRODUCTS AFTER EXPOSURE TO ALFALFA- OR CORN-SILAGE WITH**  
**DIFFERENT AMOUNTS OF ACIDITY**

## INTRODUCTION

Performance among lactating cows fed corn-based diets can be limited by metabolizable Lys (NRC, 2001). Thus, it is not surprising that increases in metabolizable Lys often increases milk secretions and augment yields of milk fat, and milk protein (Xu et al., 1997; Socha et al., 2005; Robinson et al., 2011; Arriola Apelo et al., 2013; Giallongo et al., 2016). Amino acid requirements of cattle can be met by increased intake of feeds containing greater amounts of limiting AA, or by supplementation of ruminally protected AA products (**RPAA**). Unfortunately, supplementation of feeds that do not exactly match the AA profile of requirements by cattle can result in increases in excretion of wasteful N (e.g., urine, feces). An advantage of direct supplementation of RPAA to meet limiting AA requirements of cattle is that amounts of N used for productive purposes (i.e., milk or body protein synthesis) can be increased without concomitant increases in N emissions (Bach et al., 2005; Socha et al., 2005; Wang et al., 2010; Lee et al., 2015). A myriad of feed technologies (e.g., hydroxy analogs of AA, heat-treated proteins, pH-sensitive polymeric coated AA, lipid coated AA) exist to provide RPAA to ruminants (Wu and Papas, 1997). To be an effective source of metabolizable AA, RPAA products must withstand mixing, innate chemical characteristics of the diet, mastication and ruminal fermentation (Wu and Papas, 1997). Ji et al. (2016) reported mechanical mixing, diet moisture content, and increased amounts of time exposed to feed decreased Lys retained in several lipid coated ruminally protected Lys products (**RPLys**). Hydrolysis of triglycerides to fatty acids and glycerol can be catalyzed by acid (Bender et al., 1961; Carey, 2003) and acid-catalyzed hydrolysis is greater in environments with large water concentrations (Le Chatelier, 1884; Bender et al., 1961; Carey, 2003). Thus, it is possible

that diets containing large amounts of silage and concomitantly, large amounts of water and organic acids may impact amounts of AA retained by lipid coated RPAA. However, data are limited on effects of diet acidity and amounts of Lys retained in lipid coated RPLys. Moreover, few data are available on effects of silage source (e.g., alfalfa, corn) on amounts of Lys retained by lipid coated RPLys. Therefore, our objective in this work was to determine impacts of chemical characteristics of diet on Lys retained by lipid coated RPLys, estimate ruminal degradation of Lys retained by lipid coated RPLys after mixing with alfalfa- or corn-silage with different acidity, and measure bioavailability of Lys from lipid coated RPLys after mixing with silage.

## **MATERIALS AND METHODS**

All procedures involving the use of animals were approved by the South Dakota State University Institutional Animal Care and Use Committee.

### ***Exp. 1***

We conducted an experiment to evaluate amounts of Lys retained by 2 lipid coated RPLys products after exposure to alfalfa- or corn-silage with different pH. Prior to in situ incubation of RPLys in silage, alfalfa (50% DM; 741 kg) was collected, wilted, and chopped (average particle size = 0.81 cm) with a hammer mill (Patriot Pro-Series, Model CSV – 3090H; Patriot Products Inc. Pewaukee, WI) before it was packed ( $0.7 \pm 0.05$  kg/L) into 2 miniature silos (121 L) and ensiled for 169 d. Similarly, freshly chopped (average particle size = 0.79 cm) corn plants (46% DM; 334 kg) were collected and packed ( $0.6 \pm 0.05$  kg/L) into 2 miniature silos (121 L) and ensiled for 154 d.

After ensiling, corn silage ( $\text{pH} = 3.7 \pm 0.1$ ) and alfalfa silage ( $\text{pH} = 4.4 \pm 0.1$ ) were composited by silage type and pH was measured immediately prior to in situ incubation of RPLys in silage. Silage pH was measured using a modified method of Buchanan-Smith and Yao (1981). Briefly, pH of each composited silage was measured by mixing 40 g of silage with 360 g of distilled de-ionized water; silage and water were mixed for 30 sec prior to measurement with a pH meter (Orion 3 STAR, Thermo Electron Corp). After initial pH measurement, silage pH was adjusted by mixing (model 2030, Marion Mixer, Marion, IA) 10% NaOH (wt/wt) for 5 min to achieve the appropriate acidity. Acidity in an aliquot of corn silage (11 kg DM) was modified to be similar to the initial pH of alfalfa silage ( $\text{pH} = 4.6$ ) by addition of 1.55 kg NaOH. Subsequently, an aliquot of alfalfa silage (9.5 kg DM) was mixed with 2.80 kg of NaOH to increase pH to 6.8. Additionally, another aliquot of corn silage (11 kg DM) was mixed with 3.15 kg of NaOH to achieve a similar pH ( $\text{pH} = 6.9$ ) to alfalfa silage with added NaOH.

Samples (4 g) of 2 lipid coated RPLys products were placed in polyethylene bags ( $10 \times 20$  cm, pore size =  $50 \mu\text{m}$ ; Dacron, Ankom Technology, Fairport, NY) and heat sealed prior to placement in silage. One lipid coated RPLys product (EB; LysiPEARL, Kemin Industries, Des Moines, IA) consisted of 47.5% Lys-HCl and 52.5% lipid (Table 2.1) and was manufactured by extrusion into small particles. The second lipid coated RPLys (EC; USA Lysine, Kemin Industries) consisted of 65% Lys-HCl and 35% lipid (Table 2.1) and was manufactured by extruding a Lys and lipid mixture to small particles. Subsequently, the Lys-lipid particle was then re-encapsulated with lipid. Lysine-HCl (4 g) alone served as a negative control. Additionally, Lys-HCl and lipid were added separately to the same polyethylene bags in amounts identical to either EB or EC, and

served as controls to evaluate manufacturing method. After silage acidity was adjusted polyethylene bags were hand-mixed with silage and incubated for 0, 6, 12 or 24 h. At the appropriate time, bags were removed and rinsed with 5 L of cold tap water per side over a 40  $\mu$ m screen. After rinsing, samples were frozen (-20 °C) and lyophilized prior to removal from polyethylene bags. Subsequently, RPLys were observed by a scanning electron micrograph (Hitachi S-3400 N) after coating a subsample of RPLys with Au (10 nm).

Lipid associated Lys content was analyzed from lyophilized samples as described by Brake et al. (2013). Triacylglycerols and free fatty acids were dissolved through addition of 9 mL of hexane:methanol (1:2 vol/vol) in a 50 mL screw top centrifuge tube. Tubes were vortexed for 30 sec every 5 min for 15 min and placed into an ice bath for 15 min to allow separation of the hexane and methanol layers. Subsequently, 3 mL of cold hexane:methanol was added. Tubes were centrifuged ( $1,500 \times g$  at 4 °C) for 10 min and the hexane layer collected. Cold hexane (6 mL) was then added with 6 mL of cold 0.3% (wt/wt) NaCl containing 1 mM norleucine. Tubes were centrifuged ( $1,500 \times g$  at 4 °C) for 10 min and the resulting hexane layer composited with the previously removed hexane phase. Phospholipids in the remaining aqueous phase were removed with addition of 16.5 mL chloroform:methanol (2.5:3 vol/vol), and tubes were vortexed for 10 sec. Following additions of chloroform:methanol, 10 mL of 0.3% NaCl with 1 mM norleucine was added, and tubes were vortexed for another 10 sec. After centrifugation ( $1,500 \times g$  at 4 °C) for 10 min, the subsequent chloroform layer was removed. To ensure recovery of Lys, 10 mL of 0.3% NaCl solution was then added to the chloroform layer, vortexed (10 sec) and centrifuged ( $1,500 \times g$  at 4 °C) for 10 min, and the aqueous layer was added to



the previously collected aqueous phase. The combined aqueous phase was used for determination of free Lys by HPLC. Chromatography was achieved on a Li cation-exchange column ( $4.0 \times 100$  mm; Pickering Laboratories, Inc., Mountain View, CA) at a continuous flow rate of 0.3 mL/min after passing a Li guard column ( $2.0 \times 20$  mm; Pickering Laboratories, Inc.). The initial mobile phase (32 min) had a pH of 2.75, then a mobile phase at pH 3.37 was pumped for 18 min, and a final eluent (pH 7.50) was used for 33 min. Between samples, the column was regenerated by pumping LiOH (0.4% wt/vol) for 7 min, and then reequilibrated to the first carrier solution for 45 min. Column temperature was initially maintained at 38 °C (50 min) and then heated to 63 °C for 35 min before cooling at a constant rate over 50 min to reach 38 °C. After elution from the column, AA were derivatized with *o*-phthalaldehyde (OPA) in a short coil at 50 °C before fluorescence was measured (excitation at 330 nm and emission at 465 nm) and Lys was quantified with reference to norleucine.

**Calculations.** Linear and nonlinear models were used to predict rates of Lys disassociation from lipid coated RPLys after mixing with silage. The linear model was modified from others (Mathers and Miller, 1981; Bach et al., 1998) evaluation of ruminal protein disappearance; rate of Lys disassociation was calculated as the slope of the regression line of the natural logarithm of lipid associated Lys in lipid coated RPLys vs incubation time. Additionally, we also evaluated a modification of the first-order kinetic model described by Ørskov and McDonald (1979) to predict rates of Lys disassociation:

$$\text{Lys disappearance (\%)} = \text{ID} + \text{SD} \times (1 - e^{-k_d \times t})$$

where ID is the proportion of Lys that immediately disassociates from lipid coated RPLys, SD is the proportion of Lys that is potentially dissociable,  $k_d$  is the rate of

disassociation ( $\text{h}^{-1}$ ) of SD and  $t$  is the time of exposure to silage. The equation was fitted using the Marquardt method for iterative, nonlinear, least squares estimation in SAS (SAS Inst. Inc., Cary, NC). Incorporating lag time into the model did not seem appropriate from the shape of the degradation curves (Figure 2.1). Because a coefficient of determination is not readily defined in a nonlinear regression we calculated pseudo-coefficients of determination as  $1 - (\text{sum of squared residuals} / \text{corrected total sum of squares})$ .

Independent of the mathematical model used to predict rates of Lys disassociation from lipid coated RPLys after mixing with silage, extent of Lys disassociation was calculated as (Ørskov and McDonald; 1979):

$$\text{Extent of Lys disassociation} = \text{ID} + \text{SD} \times \left( \frac{K_d}{K_d + K_p} \right)$$

where ID is the proportion of Lys that immediately disassociates from lipid coated RPLys, SD is the proportion of Lys that is potentially dissociable,  $K_d$  is the rate of disassociation ( $\text{h}^{-1}$ ) of SD and  $K_p$  is the proportional rate of diet intake ( $\text{h}^{-1}$ ). Because rate of diet intake was assumed to be constant across time (Hart et al., 2014; Yuan et al., 2015),  $K_p$  was calculated as the reciprocal of 8-, 12-, 16- and 24-h, respectively. Subsequently, amounts of Lys that remained associated with lipid coated RPLys was calculated as  $1 - \text{extent of Lys disassociation}$ .

**Statistical Analyses.** Both the linear and nonlinear model were tested for goodness of fit by the lack-of-fit test described by Sokal and Rohlf (1969). Additionally, appropriateness of the linear model was evaluated by plotting residuals vs. fitted values and coefficients of determination were calculated with REG procedure of SAS (SAS Institute Inc., Cary, NC). Pool sizes (ID, SD and ND) and  $K_d$  were analyzed as a

completely randomized design using the MIXED procedures of SAS. The model contained effects of lipid coated RPLys, silage type, pH and all interactions.

### *Exp. 2*

We conducted an additional experiment to estimate ruminal degradation of lipid associated Lys in RPLys with or without monensin. Samples (4 g) of EB and EC were placed in polyethylene bags (10 × 20 cm, pore size = 50 µm) and hand-mixed with an aliquot of the same alfalfa silage (pH = 4.4) and corn silage (pH = 3.7) used in Exp. 1 prior to addition of NaOH. Polyethylene bags containing EB and EC were removed after 30 min., rinsed with 5 L of cold tap water per side over a 40 µm screen, frozen (-20 °C) and subsequently lyophilized inside polyethylene bags.

Fresh ruminal contents from 2 ruminally cannulated cows fed a corn- and corn-silage based diet (Table 2.2) were collected 4 h after feeding. Ruminal contents were strained through 4 layers of cheesecloth into thermally insulated containers and immediately transported to the laboratory. Ruminal fluid was centrifuged (150 × g at 4 °C) for 5 min, and the supernatant layer was used as ruminal bacterial inocula. Ruminal bacterial inocula (10 mL) and 40 mL of McDougall's buffer (McDougall, 1948) with urea (13.3 mM urea) was dispensed into duplicate plastic centrifuge tubes (3.4 mm × 16.2 mm; Nalge Nunc International Corp., Rochester, NY) and flushed with CO<sub>2</sub>. Treatments consisted of additions of monensin (0 or 5 ppm monensin; lot no. M5273; Sigma-Aldrich Corp., St. Louis, MO), and Lys (30 mM Lys) from EB or EC exposed to silage for 30 min, Lys-HCl or Lys-HCl and lipid in amounts identical to EB or EC. Monensin was added as 25 µL of a monensin and ethanol solution prepared by dissolving 100 mg of

monensin in 10 mL of anhydrous ethanol. Ruminal inocula and McDougall's buffer alone served as the negative control. Fermentation tubes were capped with butyl rubber stoppers fitted with Bunsen valves, vortexed and incubated at 39 °C for 48 h. At 0, 6, 12, 24, 30, 36, 42 and 48 h fermentation tubes were vortexed and an aliquot of fluid (1 mL) was removed to a microcentrifuge tube and acidified with 0.2 mL of 1 M HCl before freezing (-20 °C). Subsequently, each aliquot of fermentation fluid was analyzed for ammonia concentration (Broderick and Kang, 1980).

**Statistical Analyses.** Ammonia concentration in fermentation fluid was analyzed as a randomized complete block design with the MIXED procedure of SAS; the model included effects of time, treatment, monensin, and all interactions; effect of cow was random. Denominator degrees of freedom were calculated by the Kenward and Roger adjustment (Kenward and Roger, 1997). Linear and quadratic effects of time were determined by orthogonal contrasts. When the *F*-statistic was significant ( $P \leq 0.05$ ), means were separated using a Student's *t*-test.

### ***Exp. 3***

Nine abomasally cannulated ewes ( $70.1 \pm 5.2$  kg;  $5.3 \pm 0.6$  yr) were used in an experiment to measure Lys bioavailability from feeding EB, EC and Lys-HCl to ruminants by a slope-ratio analysis (Roach et al., 1967; Finney, 1978; Batterham et al., 1979; Elwakeel et al., 2012). Ewes were individually housed (1.8 m  $\times$  0.72 m) in a temperature (18 °C) and light (16 h light) controlled room, and were limit-fed ( $1.6 \times \text{ME}_m$ ) twice daily (0730 and 1930 h). The diet was designed to meet or slightly exceed maintenance requirements for protein and energy (NRC, 2007). Diet composition is

reported in Table 2.3. Samples of the complete diet were taken daily and composited by period and immediately frozen at -20 °C.

Ewes were surgically fitted with abomasal catheters as described by Freetly et al. (2010) at least 14 d prior to the experiment to allow abomasal infusion of Lys. Briefly, abomasal catheters were constructed in the laboratory using 1 m of flexible polymer tubing (0.8 cm o.d., 0.5 cm i.d.; Tygon, Saint-Gobain Performance Plastics, Akron, OH) that had a pair of cuffs constructed from slightly larger flexible polymer tubing (1.0 cm o.d., 0.5 cm i.d.) placed 2.54 cm apart to create a 5 cm tip. Subsequently, catheters were surgically placed by dissection of the fundus under general anesthesia using isoflurane, and the tip of catheters were inserted in the abomasum and a purse string suture was placed between each cuff. Catheters were exteriorized 1 cm ventral to the transverse process of the L3 vertebra, and a permanent cuff (1.0 cm o.d., 0.5 cm i.d.) was fixed to the external portion of the catheter to prevent retraction into the abdominal cavity.

Ewes were placed in a  $9 \times 9$  Latin square and treatments were 0, 5, or 10 g/d of Lys from EB, EC, or Lys-HCl mixed with diet 30 min prior to feeding. Additions of EB, EC, or Lys-HCl to the diet was divided evenly between feedings. Abomasal infusion of 0, 5, or 10 g/d Lys acted as a positive control. Abomasal Lys was prepared and delivered daily in 2.6 L of distilled water via a peristaltic pump. When ewes did not receive abomasal Lys infusion 2.6 L/d of distilled water alone was abomasally infused. Each period consisted of 7 d; 6 d for adaptation and measures of plasma AA were collected on d 7 via jugular venipuncture 4 h after the morning feeding. Blood samples were placed on ice after collection, immediately transferred to the laboratory and plasma was harvested by centrifugation ( $2,200 \times g$  at -4 °C) for 15 min. Plasma was stored frozen at -20 °C until

subsequent analysis of Lys via HPLC. Ewe weights were collected immediately following blood collection and used to calculate the subsequent periods daily feed offering.

Prior to analyses of DM, OM, CP, NDF, ADF, and ether extract, diet samples were thawed at room temperature (18 °C) and subsequently dried (55 °C) in a forced-air oven for 48 h before ground to pass a 1 mm screen (Thomas-Wiley laboratory mill model 4; Thomas Scientific USA, Swedesboro, NJ). Dry matter content was determined by drying samples at 105 °C for 24 h in a forced-air oven. The wet chemistry techniques of Van Soest et al. (1991) were used to quantify NDF and ADF non-sequentially; diet NDF was measured in the presence of sodium sulfite and with addition of  $\alpha$ -amylase. Diet samples were analyzed for ether extract (procedure AM 5-04; AOCS, 2005; Ankom XT10; Ankom). Nitrogen content of diet samples were determined by combustion (method 968.06; AOAC 2012; Elementar Rapid MAX N exceed; Elementar, Langenselbold, Germany), and CP was calculated as  $6.25 \times N$ . Amino acid concentration of diet samples was analyzed by HPLC (method number 982.30; AOAC, 2006).

Plasma Lys content was analyzed for free Lys by reversed phase HPLC after pre-column derivatization of AA with OPA (Dai et al., 2014). Chromatography was achieved on a C<sub>18</sub> column (3.0  $\times$  150 mm, 3.5  $\mu$ m; Agilent Corp, Santa Clara, CA, USA) after passing a C<sub>18</sub> guard column (2.1  $\times$  12.5 mm, 5  $\mu$ m). The combined flow rate of the mobile phase was constant and 0.64 mL/min. The initial mobile phase (A) was composed of water containing 9.87  $\mu$ mol/L Na<sub>2</sub>HPO<sub>4</sub>, 18.89  $\mu$ mol/L Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, and 0.49  $\mu$ mol/L NaN<sub>3</sub>. The second mobile phase (B) was composed of water containing 8.58 mol/L acetonitrile and 11.11 mol/L methanol. The percentage of Solvent A was as follows: 0

min, 98%; 20 min, 43%; 20.1 min, 0%; 23.6 min, 98%. The column was maintained at 40 °C and injection volumes were 16 µL. Amounts of plasma Lys were quantified in reference to norvaline and measured at 338 nm (bandwidth = 10 nm) and the reference wavelength was 390 nm (bandwidth = 20 nm) with a diode array detector (Ultimate 3000; Thermo Electron North America, West Palm Beach, FL).

**Calculations.** Slopes for each treatment were calculated by regressing plasma Lys and amounts of Lys provided in feed or abomasally infused. Subsequently, Lys bioavailability was calculated as the ratio of the slopes of EB, EC or Lys-HCl and abomasal Lys infusion (Roach et al., 1967; Finney, 1978; Batterham et al., 1979; Elwakeel et al., 2012).

**Statistical Analyses.** Plasma Lys was regressed on amounts of Lys added from EB, EC, Lys-HCl or abomasal infusion using the REG procedures of SAS. Plasma AA concentration was analyzed as a Latin square using the MIXED procedure of SAS; the model included effects of treatment and effects of animal and period were considered random. Denominator degrees of freedom were calculated by the Kenward and Roger adjustment (Kenward and Roger, 1997). Effects of added Lys to feed from EB, EC, Lys-HCl or abomasal infusion were determined by linear contrasts.

## RESULTS AND DISCUSSION

### *Exp. 1*

**Quality of Fit of Mathematical Approaches.** It is important that mathematical models be tested for goodness of fit before use in predicting nutrient supplies to ruminants (Bach et al., 1998). As expected, the proportion of Lys that rapidly

disassociated from the negative control (Lys-HCl alone) and each manufacturing control (i.e., lipid and Lys-HCl in amounts identical to EB or EC) were complete ( $100 \pm 2.5\%$ ); therefore, these data were excluded from estimations of Lys disassociation from RPLys. We calculated a moderate coefficient of determination ( $r^2 = 0.56$ ) when we linearly regressed the natural logarithm of residual Lys in lipid coated RPLys and incubation time in silage. Further, analysis of residuals from the linear model suggested that a linear model was not appropriate (Figure 2.2) in estimating rates of Lys disassociation from lipid coated RPLys. Residuals should be equally distributed about a fitted line without any pattern when linear estimations are appropriate; however, linear estimation of Lys disappearance from lipid coated RPLys incubated in silage resulted in a U-shaped distribution of residuals (Figure 2.2). Additionally, the linear model was rejected by the lack-of-fit test (Sokal and Rohlf, 1969). Alternatively, estimates of Lys disappearance from lipid coated RPLys incubated in silage by a first-order kinetics model appeared to have a better fit (average pseudo- $r^2 = 0.87$ ) than linear estimates, and did not fail the lack of fit test. Ostensibly, a nonlinear model more appropriately estimated Lys disappearance across time from lipid coated RPLys incubated in silage.

***Lysine Disassociation Kinetics.*** Few data are available on effects of diet characteristics and amounts of Lys retained by lipid coated RPLys after exposure to feed. Ji et al. (2016) reported that mixing and diet DM decreased Lys retained by lipid coated RPLys. Generally, lipid coated RPLys is manufactured by mixing heated lipid and Lys. Subsequently, the mixture of Lys and lipid is cooled and manufactured to small particles. This method of manufacture likely contributes to an even distribution of Lys and lipid throughout the Lys-lipid particle. Ørskov and McDonald (1979) reported that proteins fed



to ruminants have different rates of ruminal degradation and that proteins can be characterized into 3 different fractions: 1) a rapidly soluble protein fraction that is instantly degradable, 2) a slowly degraded fraction that is degraded at a constant rate, and 3) a fraction not ruminally degraded. Subsequently, these authors (Ørskov and McDonald, 1979) proposed a first-order kinetics model to describe ruminal degradation of protein. Differences in ruminal degradation of protein is likely related to different physical characteristics of proteins (e.g., hydropathy, solubility, molecular charge). Similar to proteins, differences in physical characteristics among Lys molecules in Lys-lipid particles may allow for different rates of Lys disassociation from lipid coated RPLys. It is reasonable that Lys located at the peripheral surface of the Lys-lipid particle more rapidly disassociates in comparison to Lys embedded nearer to the core. Further, Le Chatelier (1884) established that lipids can be hydrolyzed when exposed to environments with acids and water (e.g., silages). Hydrolysis of the outer layer of lipid in the Lys-lipid particle could allow for subsequent solubilization of Lys nearer to the core of the Lys-lipid particle than Lys associated with the periphery.

***Lysine That Immediately Disassociated.*** We did not observe an interaction among product, silage, and pH on Lys disassociation from RPLys, and Lys disassociation kinetics are reported in Table 2.4. Interestingly, amounts of Lys that immediately disassociated from RPLys were different between alfalfa silage and corn silage at different amounts of acidity (*Silage*  $\times$  *pH*;  $P = 0.02$ ). Specifically, Lys that immediately disassociated from RPLys increased in corn silage as pH increased, but amounts of Lys that immediately disassociated from RPLys in alfalfa silage did not differ and were numerically less when alfalfa silage was nearer a neutral pH. Addition of 10% NaOH to

silage had little impact on DM content of low (corn silage = 31.3% DM; alfalfa silage = 41.7% DM) or high (corn silage = 30.1% DM; alfalfa silage = 37.6% DM) pH silage. Additionally, Ji et al. (2016) reported that greater diet moisture content had no effect on amounts of Lys that disassociated from similar RPLys. Indeed, our data together with the report of Ji et al. (2016) seem to suggest that acidity of silage-based diets may have a larger impact than diet moisture content. Overall, Lys that immediately disassociated from EC (15.7%) was greater ( $P = 0.04$ ) than EB (4.9%). There are limited data available on Lys disassociation from EB and EC after exposure to silage. Clearly, differences in manufacture of RPLys can impact Lys disassociation from lipid coated RPLys after exposure to silage.

***Lysine That Slowly Disassociated.*** Amounts of Lys that slowly disassociated from RPLys was less in corn silage near a neutral pH compared to acidic corn silage, and amounts of Lys that slowly disassociated from alfalfa silage was not impacted by acidity ( $Silage \times pH = 0.01$ ). Overall, 246% more Lys slowly disassociated from RPLys exposed to acidic corn silage than from RPLys exposed to neutral corn silage ( $P = 0.01$ ). We posit that RPLys exposed to acidic corn silage may be more susceptible to acid-catalyzed hydrolysis of triglycerides. Greater extent of acid-catalyzed hydrolysis of triglyceride in RPLys could allow greater amounts of Lys in the Lys-lipid particle to be dissolved by water in silage. However, acidity did not affect ( $P = 0.40$ ) Lys that slowly disassociated from RPLys mixed in alfalfa silage. Perhaps, a lack of effect of acidity on amount of Lys that slowly disassociated from RPLys in alfalfa silage was related to greater abrasion of RPLys by alfalfa silage during mixing. Indeed, RPLys incubated in alfalfa silages (Figure

2.3) often appeared to have a more abraded coating than RPLys incubated in neutral corn silages (Figure 2.4) or RPLys prior to mixing with silage (Figure 2.5).

Greater amounts of Lys slowly disassociated from EB (67.5%) compared to EC (46.7%;  $P = 0.01$ ). Obviously, differences in manufacture of RPLys can impact amount of Lys that disassociate after mixing with silage. It is possible that differences in manufacture contribute to a more uniform dispersion of Lys across the Lys-lipid particle in EB compared to a greater amount of Lys nearer the core in EC. If Lys more distal to the core of RPLys disassociates sooner after mixing with silage than Lys nearer the core of RPLys, then this may allow for a greater amount of more slowly dissociable Lys in EB.

***Lysine Not Disassociated.*** Lysine that did not disassociate by 24 h ( $32.5 \pm 5.4\%$ ; Table 2.4) did not differ between type of RPLys ( $P = 0.30$ ) and was not affected by silage ( $P = 0.64$ ). However, greater acidity reduced amounts of Lys that did not disassociate from RPLys by nearly 49%. Ji et al. (2016) reported that less Lys disassociated from EB and EC 24 h after mixing in TMR compared to our estimates. Many factors may have contributed to differences between the report of Ji et al. (2016) and our observations. Ji et al. (2016) mechanically mixed RPLys with TMR containing different amounts of DM, but we hand-mixed RPLys into 2 different silages with different amounts of acidity. Indeed, TMR in the report of Ji et al. (2016) contained alfalfa- and corn-silage, protein meal, cereal grain, vitamins, minerals, and had a pH that was intermediate to our acidic and neutral silages. Nonetheless, the observations of Ji et al. (2016) and our data provide strong evidence that Lys associated with lipid coated RPLys can be affected by mechanical forces and chemical characteristics of feed.

***Rate of Lysine Disassociation.*** At least several physical (Block and Jenkins, 1994; Ji et al., 2016) and chemical characteristics of diet can impact Lys loss from RPLys. Rate of Lys disassociation from RPLys was 10.8% per h, and we observed no differences between each RPLys or effect of silage source or acidity on rate of Lys disassociation ( $P \geq 0.21$ ), which seems to suggest that differences in RPLys manufacture and effects of silage and acidity had a greater impact on amounts of Lys able to slowly disassociate from RPLys than rate of Lys disassociation. Typically, silage acidity is stable for 4 to 6 d after exposure to environment (Dolci et al., 2011) and it seems unlikely that pH in acidic silages increased within 24 h. Clearly, producers and nutritionists should evaluate impacts of physical and chemical characteristics of diet when incorporating RPLys to allow improved estimates of metabolizable Lys from diets.

***Estimated Lysine Loss Across Several Common Feeding Intervals.*** If cows are fed multiple times daily, then feed delivery may be representative of an 8-, 12-, or 16-h feeding interval. Alternatively, feed may only be delivered once daily. If amounts of feed delivered closely matches DMI and meal intervals are evenly dispersed across 24-h (Hart et al., 2014; Yuan et al., 2015), then different feed delivery intervals can impact amount of time RPLys may be exposed to silage. Therefore, we estimated extent of Lys disassociation from RPLys (Table 2.4) at 8-, 12-, 16-, and 24-h feeding intervals assuming a constant intermeal interval among cows. Estimates of extent of Lys disassociation from RPLys reflected effects of silage source and acidity among different RPLys. Because Lys disassociated from RPLys after mixing with silage, estimates of extent of Lys disassociation from RPLys were less for time points that would reflect more frequent feeding intervals. Indeed, estimates of extent of Lys disassociation from RPLys

were nearly 14% and 22% less for an 8-h feeding interval relative to 12- and 16-h feeding intervals, respectively. Further, the average estimate of extent of Lys disassociation from RPLys during the 8-h feeding interval was 31% less compared to a 24-h feeding interval. Based on our estimates of Lys disassociation, amounts of Lys consumed by cows associated with RPLys could be increased by increasing feed intervals or by including RPLys in the feeding interval expected to be consumed in a less amount of time when feeding intervals are asynchronous (e.g., feeding system with 8- and 16-h feeding intervals). However, amounts of Lys associated with RPLys when consumed by cows may differ from amounts of metabolizable Lys in RPLys.

## ***Exp. 2***

Russell (2006) described an in vitro model to estimate ruminal degradation of Lys from increases in ammonia concentration. We conducted an in vitro experiment using the method of Russell (2006) to determine ruminal degradation of RPLys after exposure to alfalfa- or corn-silage with different acidity. Additionally, in vitro estimates of Lys degradation from RPLys were conducted either with or without added ionophore to evaluate effects of monensin on amount of Lys degraded from RPLys after mixing with silage.

There was no interaction of Lys source, monensin, and time ( $P = 1.00$ ) on amounts of ammonia released from Lys. Ammonia concentration (Table 2.5) increased (*Quadratic*  $< 0.01$ ) with greater amounts of time. Similarly, others (Russell, 2005; Elwakeel et al., 2012) reported that ammonia released from Lys fermentation after 48 h was greater than ammonia released from Lys fermentation after 24-h incubations.

However, ammonia release from in vitro cultures containing Lys was not different from cultures containing no Lys until 36 h, and at 36 h after fermentations were initiated, all cultures containing added Lys had greater ammonia content than cultures containing no added Lys. After 42 h of incubation, ammonia release from Lys was greatest from EC and when lipid and Lys in amounts identical to EB were provided to cultures; EB, Lys-HCl, and lipid and Lys in amounts identical to EC were intermediate. Among cultures with added Lys, EB was greatest and Lys-HCl was least after 48 h of incubation while EC and amounts of lipid and Lys identical to either EC or EB were intermediate. Interestingly, after 48 h incubations, ammonia concentration was less in cultures containing monensin. Further amounts of ammonia released from EB and EC were not affected by mixing with either alfalfa- or corn-silage prior to in vitro incubations.

Elwakeel et al. (2012) concluded that the model of Russell (2006) might be useful in evaluating ruminal Lys degradation, but that in vitro rates of Lys degradation are dissimilar from rates of ruminal Lys degradation reported in vivo (Velle et al., 1998; Robinson et al., 2006). Indeed, in this study we were unable to detect ammonia release from added amounts of Lys until 36 h. Even though ammonia content of cultures containing added Lys increased at a quadratic rate after 36 h, amounts of ammonia released after 48 h only reflected 61% of N contained in added amounts of Lys. Similarly, Elwakeel et al. (2012) reported that 55% of N from added amounts of Lys were released after 48 h incubations with mixed ruminal fluid. We did, however, observe differences among amounts of ammonia released from different sources of added Lys despite the need for incubation periods that are likely greater than ruminal retention times.

Russell (2005) reported that extent of Lys degradation by mixed ruminal cultures is concentration dependent and that large amounts of Lys may not be degraded. Further, Russell (2005, 2006) reported that a myriad of factors can impact extent of Lys degradation by mixed ruminal microbiota in addition to saturation of the ability of bacteria to deaminate Lys. In our study, ammonia release after 48 h of incubation was greatest from cultures that had added Lys and lipid. Additionally, monensin reduced ruminal ammonia release after 48 h of incubation. Chen and Russell (1989) reported that monensin can inhibit gram-positive bacteria that rapidly ferment Lys. Subsequently, Russell (2005) suggested that *Fusobacterium necrophorum* can account for a large amount of Lys deamination in mixed ruminal cultures, but that monensin completely inhibited Lys deamination. However, little data are available on effects of monensin on ruminal escape of RPLys. Even though monensin reduced ammonia release from Lys in mixed ruminal cultures by nearly 16% after 48 h in our study, levels of ammonia release greatly exceeded amounts of ammonia released from Lys in mixed ruminal cultures that were reported by Russell (2005). Extrapolation of in vitro data to the rumen requires caution (Russell, 2006), and it remains equivocal if differences observed in the model described by Russell (2006) accurately predict relative differences among Lys degradation in the rumen. Indeed, Elwakeel et al. (2012) determined that this in vitro approach (Russell, 2006) was inadequate at assessing ruminal degradation of a Lys derivative. Thus we conducted an in vivo study to measure Lys bioavailability.

### ***Exp. 3***

Rulquin and Kowalczyk (2003) reported that abomasal infusion of graded amounts of Met and Lys to lactating cows and subsequent measurement of plasma AA is more effective than in vitro procedures determining amounts of metabolizable AA from RPAA. We used nine mature ewes fitted with abomasal catheters and fed diets designed to not be limiting in Lys to evaluate amounts of metabolizable Lys provided from RPLys or Lys-HCl after mixing with corn silage.

Plasma Lys concentrations (Figure 2.6) increased linearly ( $P < 0.01$ ) in response to abomasal infusion of Lys indicating that our model was sensitive to increases in metabolizable Lys flow. Subsequently, bioavailability of EC, EB, and Lys-HCl in feed were calculated as the ratio of the rate of plasma Lys increase from EC, EB, or Lys-HCl in feed to the rate of plasma Lys increase in response to known amounts of Lys infused to the abomasum. Our data indicate that bioavailability of Lys from EC, EB, and Lys-HCl in feed was 18.82%, 10.74%, and 11.87%, respectively. Even though each source of Lys provided a positive rate of increase among plasma Lys, rates of increases in plasma Lys from EB ( $Linear = 0.41$ ) and Lys-HCl ( $Linear = 0.36$ ) were not different from plasma Lys levels supported by diet alone. However, the rate of plasma Lys increase in response to Lys from EC tended ( $Linear = 0.15$ ) to be greater than plasma Lys from feed alone.

Effects of feeding RPLys on plasma Lys concentration and lactation performance among lactating cows fed silage-based diets are variable. Several authors (Robinson et al., 2011; Giallongo et al., 2016) reported increases in plasma Lys and milk protein content when lactating cows were supplemented with RPLys. Conversely, others (Swanepoel et al., 2010; Robinson et al., 2010; Lee et al., 2012, 2015) reported no effect of RPLys on plasma Lys and milk protein concentration. Authors have speculated



(Robinson et al., 2010; Swanepoel et al., 2010) that a lack of response among plasma Lys or milk protein may have been related to changes in partitioning of AA for physiological functions other than milk protein production or because diets were not first-limited by metabolizable Lys. Regardless of the reason for a lack of response to RPLys, it cannot be discounted that one explanation may be that amounts of metabolizable Lys from RPLys were less than anticipated. Rulquin and Kowalczyk (2003) concluded that use of RPAA may be useful to evaluate bioavailability of AA from feed. However, our data together with others (Ji et al., 2016) emphasize caution when using RPAA as a positive control in determining estimates of AA bioavailability, and that RPAA should only be used as a positive control after effects of physical and chemical characteristics of diet on availability of AA from a RPAA has been validated. Indeed, our data clearly indicate that relatively modest amounts of metabolizable Lys were realized from RPLys after mixed with corn silage.

Plasma AA concentrations are reported in Table 2.6. The only AA affected by treatment other than Lys were modest changes in Glu, Gln, and Gly. Specifically, increased intake of EC and Lys-HCl mixed with feed mitigated plasma Glu and Gly, respectively. Additionally, greater amounts of Lys from EB increased plasma Gln and greater amounts of Lys from Lys-HCl decreased plasma Gln. Furthermore, abomasal infusion augmented plasma Gly. A specific explanation for slight changes in circulating concentrations of Glu, Gln, and Gly remains unknown. Evidently, increases in both metabolizable Lys and ruminally available Lys can impact circulating AA concentrations.

## CONCLUSIONS

Bioavailability estimates indicate that RPLys can provide metabolizable Lys to ruminants, but amounts of metabolizable Lys from RPLys are affected by both physical and chemical characteristics of diet. It remains equivocal if in vitro estimates of Lys availability to ruminal microflora from RPLys are reflective of relative differences between RPLys in vivo. Nonetheless, reduced exposure of RPLys to silage-based or acidic diets may augment amounts of metabolizable Lys provided to ruminants.

**Table 2.1.** Lipid and Lys content of RPLys products

Ingredient, % DM	EB	EC	Lipid
Lys	38.0	52.0	0.0
Lipid	52.5	35.0	100.0
Lipid profile, % lipid			
C12:0	0.5	0.3	0.3
C14:0	1.4	1.3	1.3
C15:0	0.1	0.1	0.1
C16:0	58.6	58.3	58.1
C17:0	0.2	0.2	0.2
C18:0	37.7	38.5	39.2
C18:1n9c	0.4	0.3	0.1
C18:2n6c	0.1	0.2	0.0
C20:0	0.5	0.5	0.5
C24:0	0.1	0.1	0.1

**Table 2.2.** Common diet fed to ruminal fluid donor cows in Exp. 2

Ingredient	% Diet DM
Dry-rolled corn	35.30
Corn silage	25.20
Alfalfa hay	15.20
Soybean hulls	10.00
Soybean meal	9.00
MegaLac-R <sup>1</sup>	2.00
Calcium carbonate	1.20
Sodium bicarbonate	0.80
Monocalcium phosphate	0.40
Lipid coated RPLys <sup>2</sup>	0.30
Trace mineral salt <sup>3</sup>	0.30
Magnesium oxide	0.20
Trace mineral mix <sup>4</sup>	0.05
Vitamin and Se premix <sup>5</sup>	0.05

<sup>1</sup>Calcium soaps of long-chain FA (Church and Dwight Co., Princeton, NJ)

<sup>2</sup>Delivered 14.2 g/d of Lys (LysiPEARL; Kemin Industries Inc. Des Moines, IA)

<sup>3</sup>Contained 98.9% NaCl, 0.4% Zn, 0.16% Fe, 0.12% Mn, 0.029% Cu, 0.01% I, 0.004% Co.

<sup>4</sup>Contained 5.15% Zn, 2.86% Mn, 1.80% Cu, 0.18% Co. All minerals contained as AA chelates (Availa 4; Zinpro Corp., Eden Prairie, MN)

<sup>5</sup>Provided to diets (DM basis) 3,300 IU of vitamin A/kg, 2,250 IU of vitamin D/kg, 35 IU of vitamin E/kg and 0.06 mg of Se/kg

**Table 2.3.** Composition of diet fed to ewes in Exp. 3

Item	% Diet DM
Ingredient Composition, % DM	
Corn silage	50.00
Dry-rolled corn	42.81
Cane molasses	2.50
Fishmeal	2.00
Limestone	1.00
Urea	0.66
Salt	0.50
Ammonium chloride	0.50
Mineral premix <sup>1</sup>	0.03
Chemical Composition, % DM	
DM	61.12
OM	95.37
CP	11.25
NDF	34.85
ADF	15.74
EE	4.67
AA Composition, % Total AA	
Glutamic acid	16.61
Leucine	11.45
Alanine	9.01
Proline	9.00
Aspartic acid	7.40
Valine	5.31
Phenylalanine	5.16
Glycine	5.10
Lysine	4.68
Isoleucine	4.19
Arginine	4.18
Serine	4.05
Threonine	4.05
Tyrosine	3.00
Histidine	2.44
Methionine	1.88
Cysteine	1.75
Tryptophan	0.77

<sup>1</sup>Provided to diet (DM basis) 11.0 mg/kg Zn, 0.4 mg/kg Co, 44.4 mg/kg EDDI, 20.3 mg/kg Fe, 38.7 mg/kg Mn, 4.2 mg/kg Na, 5,528 IU of vitamin A/kg, 361 IU of vitamin D/kg, and 15 IU of vitamin E/kg

**Table 2.4.** Lys disassociation from RPLys when exposed to acidic (pH = 4.6) or neutral (pH = 6.8) alfalfa- or corn-silage<sup>1</sup>

Item, %	Alfalfa silage				Corn silage				<i>P-values</i>						
	EB		EC		EB		EC		Product	Silage	pH	Product × Silage	Silage × pH	Product × pH	
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH							
Degradation kinetics <sup>2</sup>															
ID	5.2	0.0	24.7	15.1	0.9	14.3	0.1	23.0	0.04	0.71	0.24	0.16	0.02	0.77	
SD	73.2	54.5	52.1	53.8	99.1	43.0	59.3	21.4	0.01	0.70	<0.01	0.18	0.02	0.19	
ND	21.7	45.5	23.2	31.1	0.0	42.7	40.7	55.6	0.30	0.64	0.04	0.11	0.50	0.27	
Kd, %/h	9.2	11.5	6.1	11.8	3.3	10.9	18.8	14.6	0.29	0.56	0.46	0.18	0.76	0.58	
Lysine loss															
8h	24.0	19.2	32.4	35.7	15.9	29.3	32.7	32.7							
12h	29.4	24.5	34.6	41.0	22.3	33.9	39.5	35.1							
16h	33.5	28.4	36.2	44.7	28.0	37.4	43.7	36.8							
24h	39.4	33.8	38.3	49.8	37.7	42.1	48.4	38.8							

<sup>1</sup>Estimated as: Lys disappearance (%) = ID + SD × (1 – e<sup>-kd × t</sup>)

<sup>2</sup>ID = Lys disassociated immediately, SD = Lys slowly disassociated, Kd = rate of Lys disassociation, ND = Lys not disassociated

**Table 2.5.** In vitro ammonia (mM) release from RPLys products, their analogs, or Lys-HCl<sup>1,2</sup>

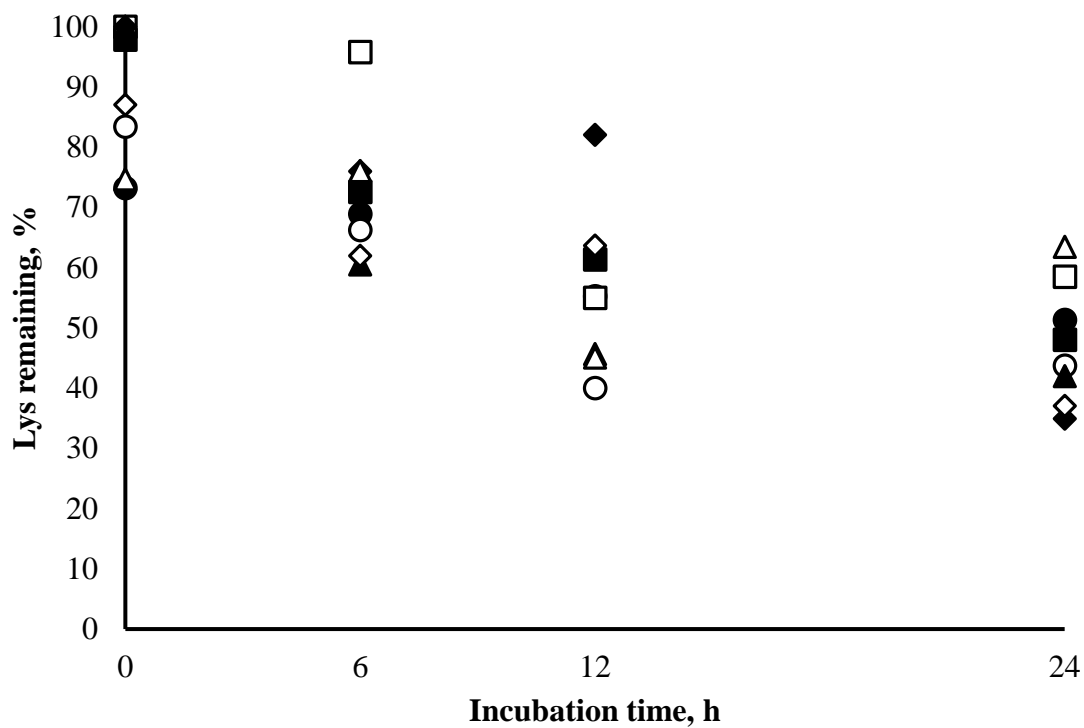
Lysine Source	Lysine, mM	Monensin, mg/L	Fermentation time, h								
			0	6	12	18	24	30	36	42	48 <sup>3</sup>
EB Alfalfa	30	0	2.69	18.03	19.93	20.30	17.02	22.25	24.08 <sup>B</sup>	30.56 <sup>BC</sup>	41.55 <sup>D</sup>
EB Corn	30	0	2.58	16.01	19.46	22.03	18.14	23.95	25.84 <sup>B</sup>	30.13 <sup>BC</sup>	44.00 <sup>D</sup>
EC Alfalfa	30	0	2.48	18.03	19.68	20.30	18.54	22.25	26.16 <sup>B</sup>	30.56 <sup>C</sup>	39.41 <sup>CD</sup>
EC Corn	30	0	2.49	16.51	19.05	20.33	17.74	23.78	24.13 <sup>B</sup>	29.51 <sup>C</sup>	41.88 <sup>CD</sup>
EB analogue <sup>4</sup>	30	0	2.51	18.63	19.86	21.82	18.19	24.39	25.61 <sup>B</sup>	29.00 <sup>C</sup>	37.38 <sup>BC</sup>
EC analogue <sup>5</sup>	30	0	2.65	17.00	20.12	22.18	18.10	23.23	23.70 <sup>B</sup>	26.55 <sup>B</sup>	38.10 <sup>BC</sup>
Lys-HCl	30	0	2.47	15.96	20.22	20.17	17.95	24.01	25.24 <sup>B</sup>	26.59 <sup>B</sup>	37.42 <sup>B</sup>
No Lys	0	0	2.59	14.98	20.27	21.06	17.94	21.53	20.84 <sup>A</sup>	20.31 <sup>A</sup>	23.31 <sup>A</sup>
EB Alfalfa	30	5	2.64	15.70	20.18	18.64	16.35	23.58	24.46 <sup>A</sup>	24.66 <sup>BC</sup>	34.36 <sup>D</sup>
EB Corn	30	5	2.48	16.67	19.62	19.37	16.96	23.95	24.97 <sup>B</sup>	26.36 <sup>BC</sup>	37.51 <sup>D</sup>
EC Alfalfa	30	5	2.54	17.38	19.03	18.42	17.30	23.04	24.91 <sup>B</sup>	29.27 <sup>C</sup>	32.47 <sup>CD</sup>
EC Corn	30	5	2.49	17.92	19.15	19.99	17.06	24.59	25.28 <sup>B</sup>	28.57 <sup>C</sup>	37.69 <sup>CD</sup>
EB analogue <sup>4</sup>	30	5	2.65	18.66	19.75	20.81	16.86	24.50	25.32 <sup>B</sup>	29.92 <sup>C</sup>	33.95 <sup>BC</sup>
EC analogue <sup>5</sup>	30	5	2.52	16.73	19.87	21.30	17.68	25.40	23.88 <sup>B</sup>	25.41 <sup>B</sup>	34.05 <sup>BC</sup>
Lys-HCl	30	5	2.54	16.77	19.32	19.10	17.61	22.95	23.11 <sup>B</sup>	25.94 <sup>B</sup>	29.39 <sup>B</sup>
No Lys	0	5	2.73	17.51	20.72	21.29	17.24	21.62	20.16 <sup>A</sup>	19.47 <sup>A</sup>	21.63 <sup>A</sup>

<sup>1</sup>Effect of hour was quadratic<sup>2</sup>Means within column lacking common superscript differ ( $P < 0.05$ )<sup>3</sup>Effect of monensin was significant ( $P < 0.01$ ) and decreased ammonia concentration<sup>4</sup>Lys-HCl and lipid added to in vitro cultures in amounts identical to EB<sup>5</sup>Lys-HCl and lipid added to in vitro cultures in amounts identical to EC

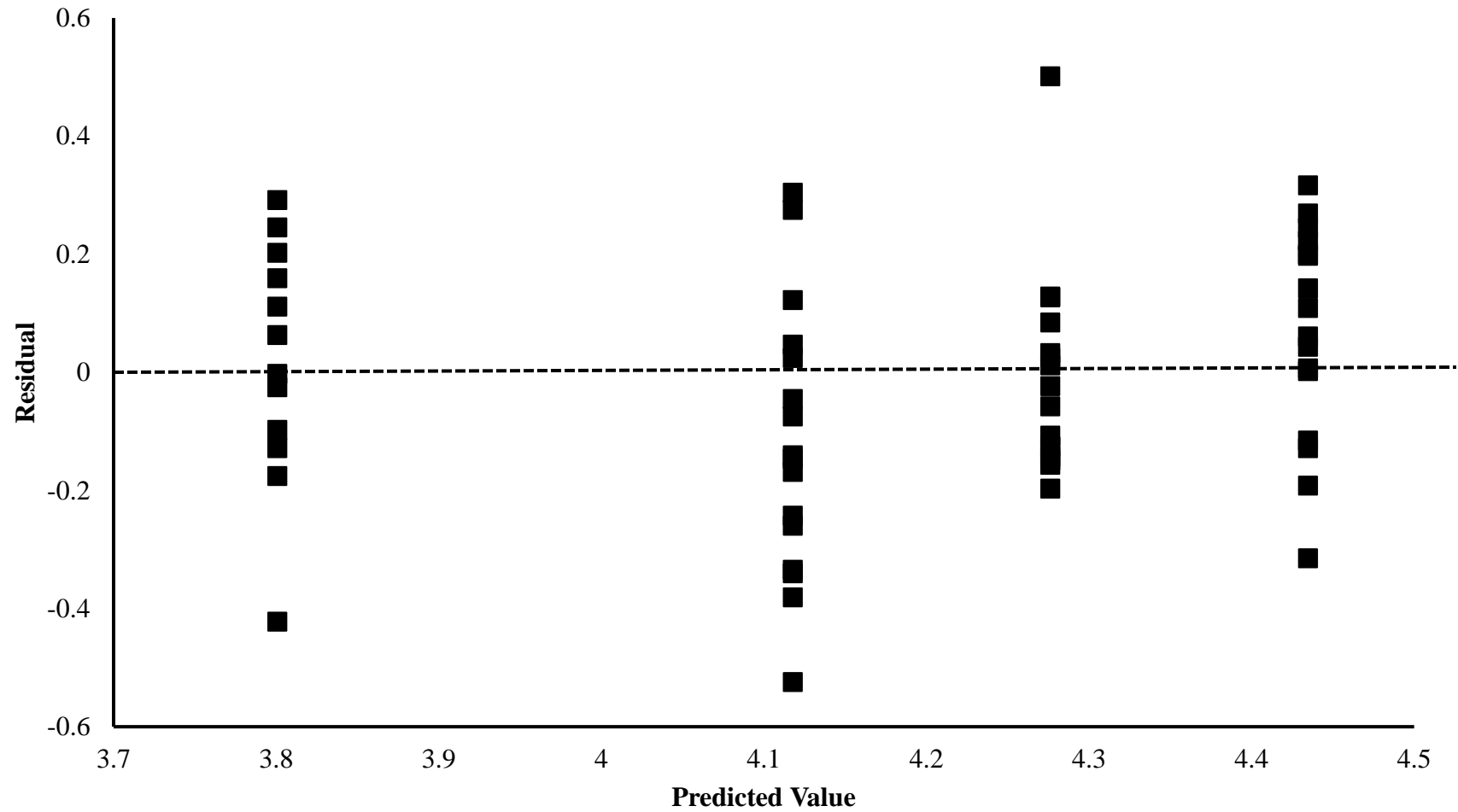
**Table 2.6.** Effect of added amount of Lys from abomasal infusion or addition of Lys-HCl and 2 lipid coated Lys products (EB, EC) on plasma AA concentrations ( $\mu\text{mol/L}$ ) of sheep

AA	Control	Infused		Lys-HCl		EB		EC		Linear contrasts			
	None	5	10	5	10	5	10	5	10	Infused	Lys-HCl	EB	EC
Glu	14.40	12.63	13.00	14.75	13.86	11.15	12.06	15.28	11.74	0.54	0.23	0.54	0.04
Asp	10.23	7.99	8.50	9.69	8.90	9.17	9.94	8.95	8.54	0.12	0.26	0.40	0.30
Ser	10.55	9.16	10.49	9.96	9.22	9.39	10.32	11.48	8.79	0.85	0.21	0.29	0.19
Gln	126.14	116.39	124.99	128.86	113.35	114.31	131.98	131.49	116.33	0.39	0.03	0.03	0.95
His	24.68	23.28	22.61	23.85	22.78	23.79	23.98	25.03	24.14	0.44	0.50	0.91	0.56
Gly	61.93	54.54	55.14	55.31	54.77	57.01	64.08	59.96	53.78	0.02	0.02	0.07	0.29
Thr	16.38	12.98	12.65	12.69	15.89	14.18	14.14	15.47	12.56	0.49	0.41	0.98	0.53
Arg	33.31	33.21	39.23	32.43	30.52	31.50	34.13	32.47	32.47	0.11	0.26	0.41	0.60
Ala	22.96	22.46	23.58	23.01	20.40	21.21	21.82	22.93	20.50	0.33	0.43	0.74	0.54
Tyr	13.06	10.66	10.14	12.28	12.38	12.26	11.51	12.21	10.47	0.16	0.37	0.43	0.47
Val	20.15	21.47	21.63	21.15	20.44	20.82	21.40	20.90	19.31	0.69	0.92	0.81	0.84
Phe	8.94	8.13	7.31	8.70	8.43	8.52	7.93	8.25	7.77	0.34	0.45	0.37	0.63
Ile	11.17	10.64	10.90	10.84	9.89	10.22	10.52	9.81	9.68	0.76	0.61	0.81	0.56
Leu	14.13	13.67	13.56	13.76	12.46	13.36	13.99	13.43	10.66	0.73	0.24	0.63	0.67

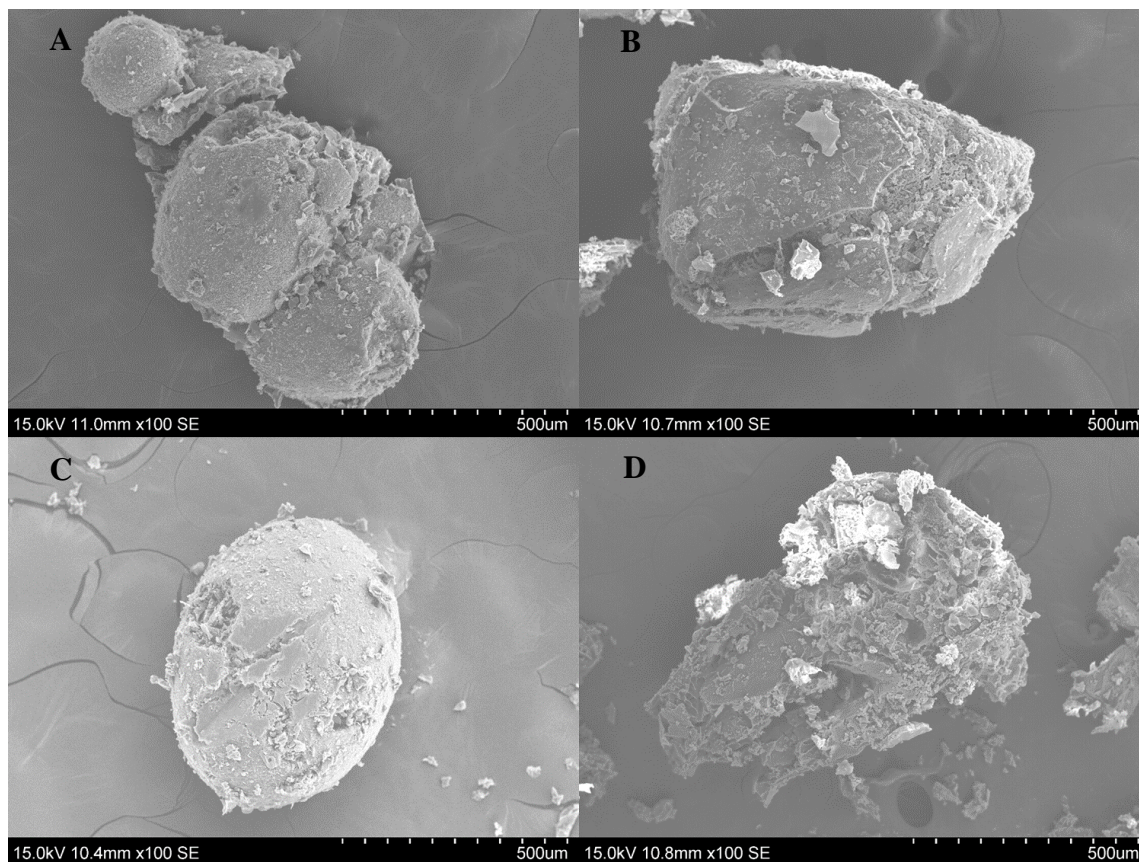




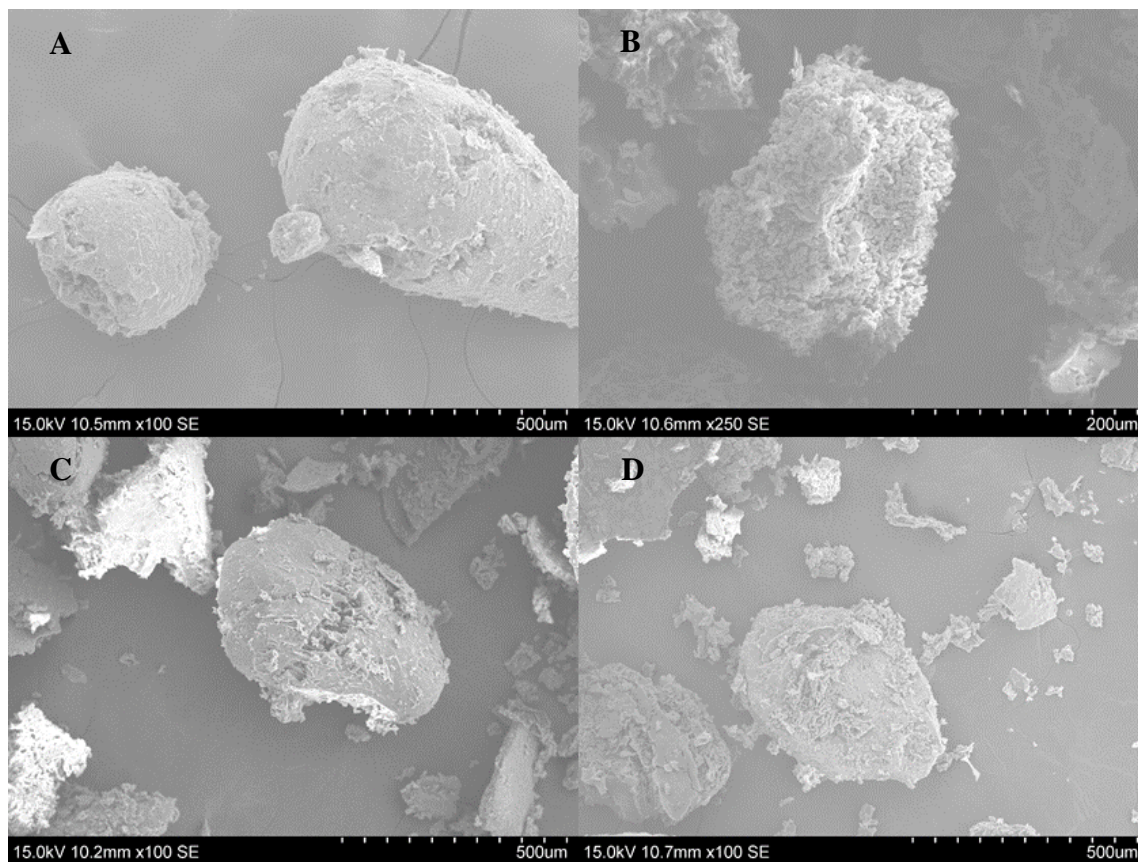
**Figure 2.1.** Lys loss from lipid coated RPLys after exposure to acidic (pH = 4.6) or neutral (pH = 6.8) alfalfa- or corn-silage. EB in acidic alfalfa silage, ■; EC in acidic alfalfa silage, ●; EB in neutral alfalfa silage, □; EC in neutral alfalfa silage, ○; EB in acidic corn silage, ◆; EC in acidic corn silage, ▲; EB in neutral corn silage, ◇; EC in neutral corn silage, △.



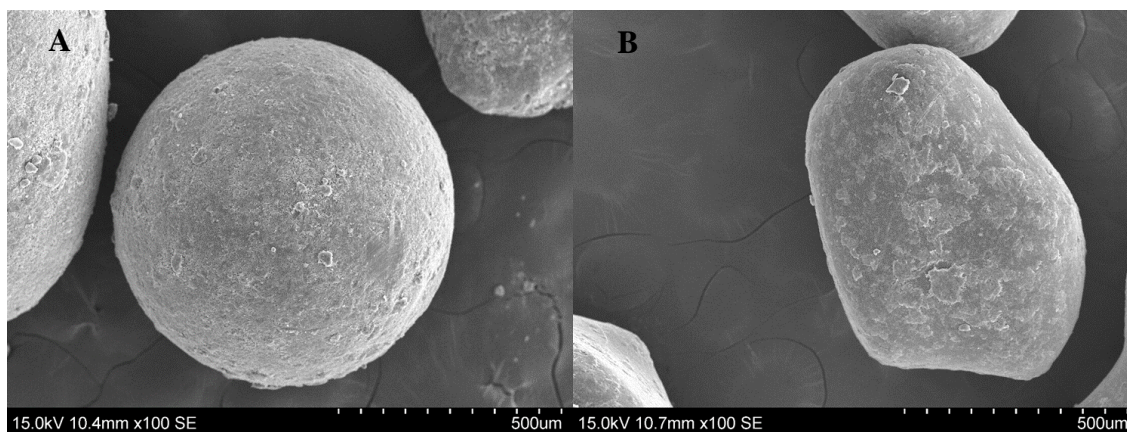
**Figure 2.2.** Pattern of Lys disassociation from RPLys as described by a linear model (Mathers and Miller, 1981) and as affected by silage incubation times included in the model.



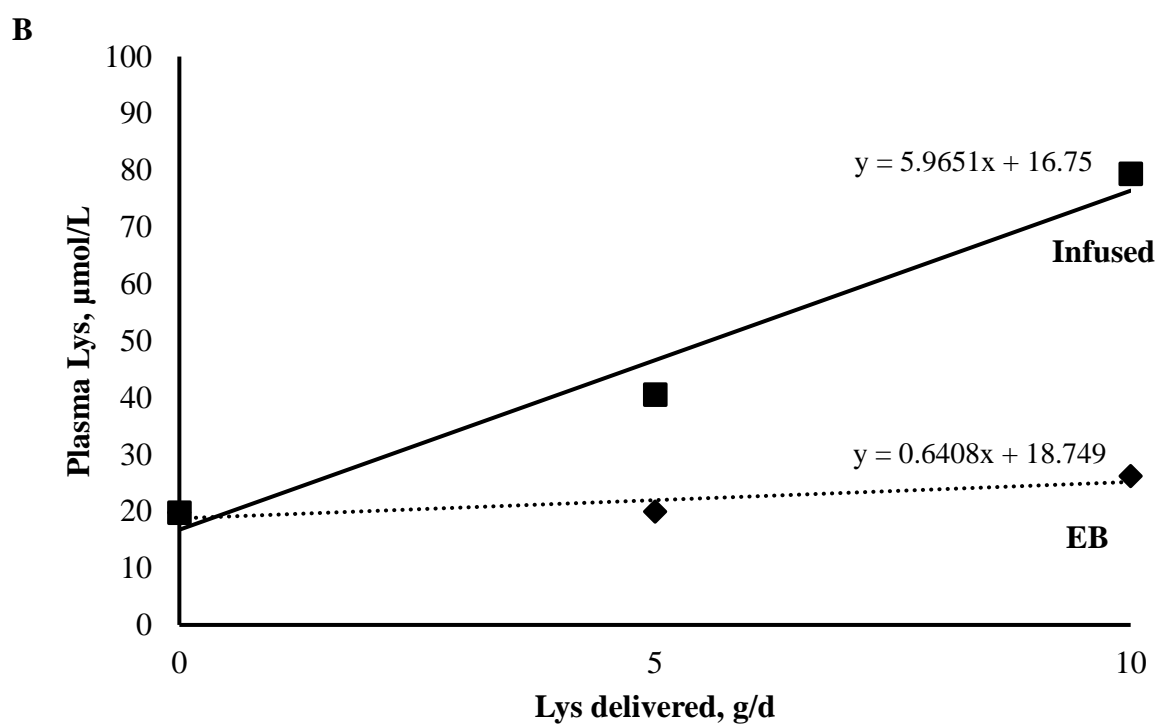
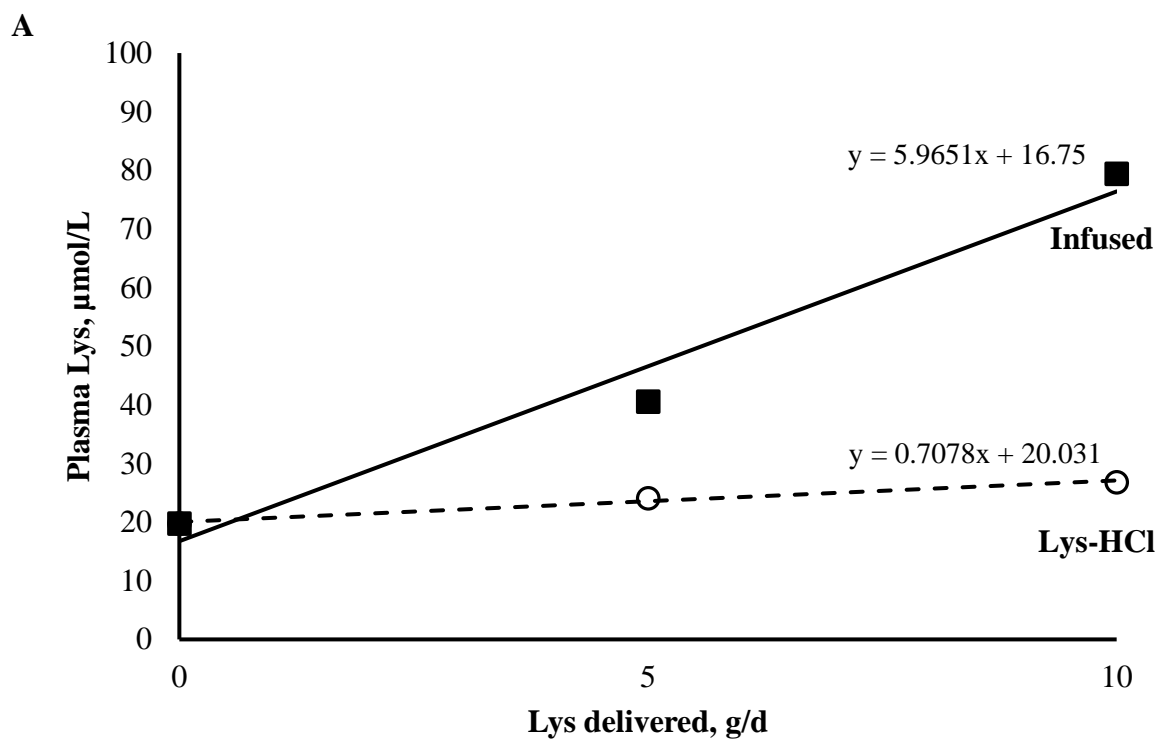
**Figure 2.3.** Scanning electron microscopy of RPLys after 24 h incubation in alfalfa silage. Panel A: Scanning electron micrograph of EB after incubation in low pH alfalfa silage. Panel B: Scanning electron micrograph of EC incubated in low pH alfalfa silage. Panel C: Scanning electron micrograph of EB incubated in neutral pH alfalfa silage. Panel D: Scanning electron micrograph of EC incubated in neutral pH alfalfa silage.

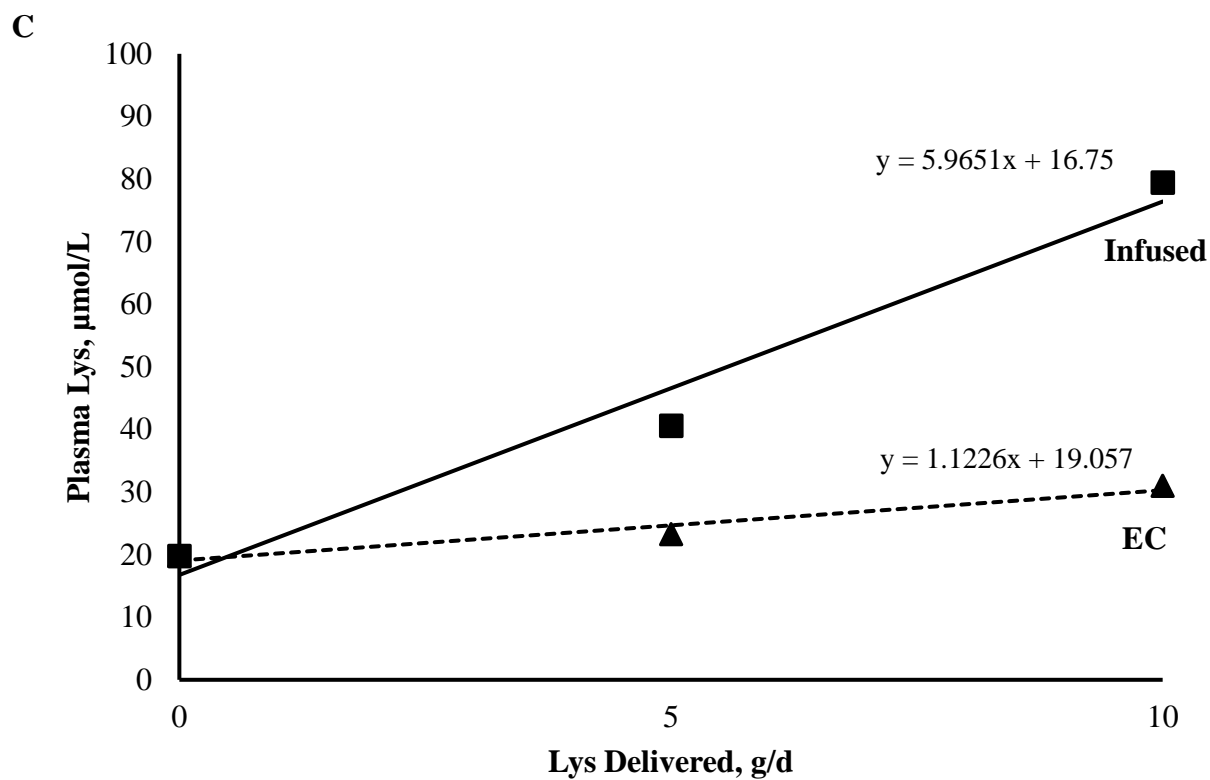


**Figure 2.4.** Scanning electron microscopy of RPLys after 24 h incubation in corn silage. Panel A: Scanning electron micrograph of EB incubated in low pH corn silage. Panel B: Scanning electron micrograph of EC incubated in low pH corn silage. Panel C: Scanning electron micrograph of EB incubated in neutral pH corn silage. Panel D: Scanning electron micrograph of EC incubated in neutral pH corn silage.



**Figure 2.5.** Scanning electron microscopy of typical RPLys product prior to incubation in silages. Panel A: Scanning electron micrograph of EB. Panel B: Scanning electron micrograph of EC.





**Figure 2.6.** Plasma Lys concentration among ewes provided Lys as abomasal infusion and as Lys-HCl (A), EB (B), or EC (C) mixed with feed. SEM = 5.6.

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